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Stimulation of neurite outgrowth using positively charged hydrogels

Mahrokh Dadsetan^a, Andrew M. Knight^b, Lichun Lu^a, Anthony J. Windebank^b, and Michael J. Yaszemski^{a,*}

^a Department of Orthopedic Surgery, Mayo Clinic College of Medicine, 200 First Street SW, Rochester MN 55905

^b Department of Neurology, Mayo Clinic College of Medicine, 200 First Street SW, Rochester MN 55905

Abstract

Autologous nerve grafts are currently the best option for the treatment of segmental peripheral nerve defects. However, autografts have several drawbacks including size mismatch and loss of sensation in the donor nerve's sensory distribution. In this work, we have investigated the development of a synthetic hydrogel that contains positive charge for use as a substrate for nerve cell attachment and neurite outgrowth in culture. We have demonstrated that modification of oligo-(polyethylene glycol) fumarate (OPF) with a positively charged monomer improves primary sensory rat neuron attachment and differentiation in a dose-dependent manner. Positively charged hydrogels also supported attachment of dorsal root ganglion (DRG) explants that contain sensory neurons, Schwann cells and neuronal support cells. Furthermore, charged hydrogels were analyzed for the appearance of myelinated structures in a co-culture containing DRG neurons and Schwann cells. DRGs and Schwann cells remained viable on charged hydrogels for a time period of three weeks and neurites extended from the DRGs. Sudan black staining revealed that neurites emerging from DRGs were accompanied by migrating Schwann cells. These findings suggest that charged OPF hydrogels are capable of sustaining both primary nerve cells and the neural support cells that are critical for regeneration.

Keywords

hydrogel; nerve regeneration; Schwann cells; scaffold

1. Introduction

The most frequently used clinical approach to repair segmental peripheral nerve defects is an autologous nerve graft. However, autografts have several drawbacks including loss of function in the donor nerve graft sensory distribution and size mismatch between the damaged nerve

*Correspondence to: Michael J. Yaszemski, M.D., Ph.D., 200 First Street SW, MS 3-69, Rochester MN 55905, Phone: 507-284-3747, Fax: 507-284-5075, Email: E-mail: yaszemski.michael@mayo.edu.

Conflicts of Interest

A non provisional patent has been filed for photocrosslinkable oligo(polyethylene glycol) fumarate used in this research, and this technology has been licensed to BonWrx.

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and the nerve graft. As an alternative to nerve autografts, a number of different natural and synthetic materials have been explored to effect nerve regeneration [1]. Although natural materials have inherent bioactivity and biocompatibility that may aid in nerve regeneration, synthetic materials offer several advantages, such as controllable physical properties, biochemical properties, and degradation rates, each of which can be tailored for specific applications. Poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and poly(lactic-co-glycolic acid) (PLGA) were some of the first synthetic polymers studied because of their availability, ease of processing, biodegradation, and FDA approval status [2–4]. Several nondegradable polymers have also been used in nerve repair applications, including silicone tubing and expanded poly(tetrafluoroethylene) [5,6]. Silicone, in particular, has been studied as a model system for nerve regeneration since the 1960s. However, guidance channels made of this impermeable, inert material do not support regeneration across defects in a rat model larger than 10 mm without the presence of exogenous growth factors [7]. Currently, attempts are being made to develop semi-permeable or degradable guidance channels that can actively stimulate nerve regeneration over longer, more clinically relevant defect lengths. Both degradable and nondegradable hydrogels have been explored for nerve regeneration applications due to their permeability and biocompatibility [8–10]. The hydrogels consist of a water-saturated polymeric network that maintains a physiological environment at the implantation site. This environment is suitable for the diffusion of trophic molecules released from the reactive tissue bordering the defect after nerve transection. In one approach, a poly(ethylene glycol) (PEG) solution combined with a crosslinkable PEG-based hydrogel has been used to approximate the epineurium of transected sciatic nerves [11]. This study demonstrated that axonal conduction can be restored following sutureless nerve repair with a polymeric material. However, this process is only applicable if the severed nerve ends are adjacent to each other, and cannot be used for segmental nerve defects. Among other hydrogels, PEG-based hydrogels have been extensively studied for their use in tissue engineering and regenerative medicine applications [8,12,13]. The drawback of PEG-based hydrogels is a low cell attachment rate as a result of the formation of a hydrated surface layer that inhibits adsorption of adhesion specific proteins such as fibronectin. Recent studies have demonstrated that modification of PEG-based systems can improve cell attachment to their surfaces. For example, PC12 cells (a neuronal cell line) extend neurites on crosslinked PEG hydrogels when the cell adhesion peptide RGDS is incorporated into the hydrogel [9]. Other investigators reported enhanced osteoblast and fibroblast attachment to PEG-based and hydroxyethylmethacrylate (HEMA) hydrogels with incorporation of positively and negatively charged monomers [14,15]. However, the effects of charge incorporation into these hydrogels on nerve cell attachment and neurite extension have not been studied.

Electric charges play an important role in stimulating either the proliferation or differentiation of various cell types. Neurite extension, for example is substantially enhanced on piezoelectric materials (i.e., materials that generate a surface charge with small deformations) such as poly(vinylidene fluoride), and on electrically conducting polymers such as polypyrrole. Neurite outgrowth occurred to a greater extent on positively charged fluorinated ethylene propylene (FEP) films in both serum-free and serum-containing media than on negatively charged or uncharged films [16]. The neurite outgrowth in that study was highly correlated to the magnitude and polarity of the charge [16]. Other investigators have also shown an enhancing effect of polycationic chitosan on embryonic chick dorsal root ganglia neurite extension [17].

Given these data, we hypothesized that oligo-(polyethylene glycol) fumarate (OPF), a biocompatible and biodegradable macromer, could be copolymerized with [2-(methacryloyloxy) ethyl]-trimethylammonium chloride (MAETAC) to produce a positively charged hydrogel as a substrate to enhance neuronal cell attachment and differentiation. We investigate the effect of MAETAC concentration on equilibrium swelling and mechanical properties of the hydrogels of different formulations. Additionally, this work evaluates the

effect of localized positive charge on neurite outgrowth in culture, with the objective that a positively charged hydrogel ultimately can be used for stimulating in vivo nerve regeneration. Neurite outgrowth from dissociated dorsal root ganglion (DRG) cells and DRG explants has been investigated in vitro. Moreover, myelin formation by Schwann cells has been studied in co-cultures of rat DRG neurons and Schwann cells on charged hydrogels.

2. Materials and Methods

2.1 Synthesis

OPF with a number average molecular weight (M_n) of $16,246 \pm 3,710$ was synthesized using PEG with an M_n of 10,000, according to a previously described method [18]. Briefly, 50 g PEG was azeotropically distilled in toluene to remove residual water and then dissolved in 500 mL distilled methylene chloride. The resulting PEG was placed in an ice bath and purged with nitrogen for 10 minutes. Then, 0.9 mol triethylamine (TEA; Aldrich, Milwaukee, WI) per mol PEG and 1.8 mol distilled fumaryl chloride (Acros, Pittsburgh, PA) per mol PEG were added dropwise. The reaction vessel was then removed from the ice bath and stirred at room temperature for 48 hours. For purification, methylene chloride was removed by rotary evaporation. The resulting OPF was dissolved in ethyl acetate and filtered to remove the salt from the reaction of TEA and chloride. OPF was recrystallized in ethyl acetate and vacuum dried overnight.

2.2. Gel Permeation Chromatography

The molecular weights of the OPF macromer and the PEG used for synthesis were measured with a Waters 717 Plus Autosampler gel permeation chromatography system (Milford, MA) connected to a model 515 high-performance liquid chromatography pump and model 2410 refractive index detector. Monodisperse polystyrene standards (Polysciences, Warrington, PA) with number average molecular weights of 474, 6690, 18600, and 38000 g/mol and polydispersities of less than 1.1 were used for the calibration curve. Three samples of each material were analyzed.

2.3. Hydrogel Fabrication

Hydrogels were made by dissolving 1g OPF macromer in deionized water containing 0.05% (w/w) of a photoinitiator (Irgacure 2959, Ciba-Specialty Chemicals) and 0.3 g N-vinyl pyrrolidinone (NVP). The OPF hydrogel was chemically modified by incorporation of a positively charged monomer, MAETAC, which is a bifunctional molecule containing both a pH-independent cationic head (quaternary ammonium) and a reactive methacryl group that copolymerizes with the fumarate group of the OPF (Fig. 1). A series of positively charged hydrogels was produced by adding MAETAC (75%, Aldrich) at different concentrations to the OPF solution (Table 1). The OPF/MAETAC mixture was pipetted between glass slides with a 1-mm spacer and polymerized by exposure to UV light (365 nm) at an intensity of 8 mW/cm^2 (Black-Ray Model 100AP) for 30 min.

2.4. Compression Testing

After crosslinking, hydrogels were cut with a cork borer into 10 mm diameter disks, and were swollen in phosphate-buffered saline (PBS, pH 7.0) for 24 hours. The compression modulus of hydrogels was determined at room temperature using a dynamic mechanical analyzer (DMA-2980, TA Instruments, New Castle, DE) in the controlled forced mode. The hydrogels were clamped between a parallel-plate compression clamp (diameter upper plate 0.6 cm, diameter lower plate 2.5 cm), and a static force was applied at a rate of 4 N/min to 18 N. The storage modulus was determined as the slope of the stress versus strain curve in its linear region at low strain (<20%).

2.5. Swelling Measurements

Ten millimeter diameter OPF hydrogel disks were vacuum dried after fabrication, weighed (W_i , initial weight) and swollen for 24 hours at 37°C in either PBS or deionized water to equilibrium swelling. Swollen samples were blotted dry and weighed (W_s , swollen weight), then dried in reduced pressure and weighed again (W_d , final dry weight). The swelling ratio and sol fraction of the hydrogels were calculated using the following equations:

$$\text{Swelling ratio} = (W_s - W_d) / W_d$$

$$\text{Sol Fraction} = [(W_i - W_d) / W_d] \times 100$$

2.6. Attenuated Total Reflectance (ATR) Fourier Transform Infrared (FTIR) Spectroscopy

The surface of OPF hydrogels with and without modification was characterized using micro ATR-FTIR spectroscopy (Nicolet 8700), coupled to a Continuum microscope (Thermo Electron Corp., Madison, WI). The microscope used an ATR slide on a germanium crystal and spectra were collected at a resolution of 4 cm⁻¹ for 128 scans with a sampling area of 150×150 μm. Multiple spectra were collected on each hydrogel surface. The differences in surface composition among the various hydrogel formulations were quantified by measuring the ratios of the characteristic peaks.

2.7. Neuronal Cultures

Procedures used in animal experiments were processed through institutional animal care and use committee at the Mayo Clinic. Dorsal root ganglions (DRGs) were excised from 6–12 E15 Sprague-Dawley rat pups (Harlan), trypsinized, and mechanically dissociated. Each pup gives 30–40 ganglions, and 10,000 DRG cells are isolated from each ganglion. The cell suspension thus obtained was plated onto hydrogels with different charges in MEM (minimum essential medium, GIBCO) supplemented with 15% calf serum for dissociated cells, or 10% calf serum for DRG explant cultures, nerve growth factor (NGF, 8ng/mL), glucose (0.6% w/v), and L-glutamine (1.4 μM; Sigma). For dissociated cell cultures, the DRG's were treated with 0.25% trypsin in Hanks balanced salt solution for 30 minutes at 37°C and disrupted through a restricted glass pipette. The DRGs were then plated on collagen-coated plates. Contaminant non-neuronal supporting cells were eliminated by treatment with 4μM of 5-fluoro-2-deoxy-uridine (FUDR) plus 4μM of Uridine (Sigma, St. Louis, MO), which was added to the media and incubated in a humidified atmosphere at 37°C, 5% CO₂ for 3 to 5 days.

2.8. DRG Explants

DRGs were dissected from E15 rat embryos, and 10–15 DRG's were plated onto each OPF hydrogel disk. The disks had varying concentrations of charged monomer. Quantification of neurite extension on the charge-modified hydrogels was performed after 24 and 40 hours using a digital image analysis system that consisted of a Zeiss Axiovert Model 35 microscope with a Nikon CCD camera. Light microscopic images of the DRGs cultured on hydrogels were recorded, and the lengths of the longest neurites from each explant were measured using NIH Image J software. The data are reported as means ± SEM.

2.9. Schwann Cell Cultures

Schwann cells were harvested from the sciatic nerves of 2 to 5 day old Sprague-Dawley rat pups, according to a previously published method [19]. Briefly, the stripped nerves were digested for 45 minutes in 0.25% trypsin/0.03% collagenase in Hanks buffer, and mechanically dissociated. The subsequent cell suspension was plated on laminin-coated Petri dishes in Dulbecco's Modified Eagle Medium/F12 (DMEM/F12, Gibco) supplemented with 10% fetal

calf serum (FCS, Gibco). The Schwann cells were grown for 48 hours to approximately 80% confluency before being trypsinized in trypsin/EDTA and counted with a hemocytometer.

2.10. Neuron-Schwann Cell Cultures

The dissociated DRG cultures, which contained both neuronal cells and Schwann cells, were not treated with FUDR, and were maintained in media that was slightly modified from the neuronal cell culture media described above. In this modified media, the calf serum was omitted, and both B27 supplement (Invitrogen) and an additional 70 µg/mL ascorbic acid were added. The media was changed 3 times a week for three weeks. Following that, the cells were fixed with 4% paraformaldehyde in PBS and the cultures were stained with Sudan Black [20].

2.11. Statistical Analysis

The data for hydrogel characterization are reported as means $\pm$ standard deviations (SD) for triplicate samples, unless otherwise described in the experimental section. Single factor analysis of variance (ANOVA) was performed (StatView, version 5.0.1.0, SAS Institute, Inc, Cary, NC) to assess the statistical significance of the results. When the global F-test was positive at the 0.05 level, Bonferroni's method was used for multiple comparison tests to determine differences among the experimental groups.

3. Results

3.1. Hydrogel Characterization

Incorporation of charged monomer into the hydrogel was analyzed using ATR-FTIR. The ATR-FTIR spectrum of unmodified, lyophilized OPF hydrogel appears in Fig. 2A. Bands at 1650 and 1085 cm^{-1} are assigned to the carbonyl and carbon-oxygen bonds (C-O-C) of OPF, respectively. After copolymerization of the hydrogel with MAETAC, a new peak emerged at 1725 cm^{-1} that is characteristic of the methacroyl carbonyl from MAETAC (Fig. 2B). Peak heights of the methacroyl group increased as the MAETAC concentration in the hydrogel formulation increased (Fig. 2B). The relative amount of MAETAC copolymerization in the OPF hydrogels was calculated by comparing the ratios of the OPF absorbance (at its characteristic peak, 1650 cm^{-1}) with the MAETAC absorbance (at its characteristic peak, 1725 cm^{-1}) in the four hydrogel formulations (Fig. 2C). According to this analysis, the amount of MAETAC that actually copolymerized within the OPF hydrogels during the crosslinking process increased linearly ($R^2=0.9905$) as the amount of MAETAC added to the formulation was increased. After hydration in either deionized water or PBS, most of the bands either broadened or disappeared. Peaks at 1725 and 1650 cm^{-1} merged together and appeared as a single band at about 1650 cm^{-1} . As seen, there were two major bands at 1650 and 1085 cm^{-1} that are assigned to carbonyl and C-O bond, respectively (Fig. 3A). To quantify the dependence of surface composition on the solvent (PBS or deionized water), the ratio of the C-O peak at 1085 cm^{-1} to the carbonyl peak at 1650 cm^{-1} was calculated for hydrogels of all formulations (Fig. 3B). After hydration in deionized water, the 1085/1650 peak ratio decreased with increasing MAETAC concentration, indicating a decrease in hydrophilic groups on the surface. However, the decrease in the 1085/1650 peak ratio for the same samples in PBS was not as pronounced as it was in water.

The swelling ratios and sol fractions of the hydrogels with four different concentrations of MAETAC are compared in Table 1. The addition of MAETAC to the hydrogels resulted in their sol fraction after swelling in water decreasing from 15.7 $\pm$ 0.8% to 9.1 $\pm$ 2.6%. The hydrogel crosslinking density increased with increasing MAETAC concentrations.

The swelling ratios of the hydrogel formulations were measured in both deionized water and PBS. The swelling ratios in deionized water increased as the concentration of MAETAC increased. For example, the swelling ratio for HG-0 (no MAETAC) was 6.2 ± 0.2 , which is lower than that for all the MAETAC containing hydrogel formulations, which had swelling ratios from 7.6 to 9.2 ($p < 0.05$). In PBS, the unmodified OPF hydrogel had a swelling ratio of 6.8 ± 0.3 , and it decreased significantly with the initial addition of MAETAC to the formulation ($p < 0.05$). However, increasing the MAETAC concentration above that initial addition did not further change the swelling ratio. Table 1 also shows that the compressive modulus of the hydrogel formulations increased significantly with the addition of MAETAC ($p < 0.05$). Unmodified OPF hydrogel had a modulus of 225 ± 20 kPa, and it increased to 331 ± 25 kPa with the addition of 200 nM MAETAC. Further addition of MAETAC did not affect the compressive modulus of the hydrogels.

3.2. DRG Explant Neurite Extension

Charged hydrogels supported the growth of DRG explants dissected from rat embryos. In addition to neurons, these explants contained Schwann cells, fibroblasts, and other neuronal support cells. Fig. 4A shows that DRG explants attached themselves to the charged surfaces and extended neurites, while unmodified hydrogels did not support neurite extension from DRG explants (Fig. 4B). Image analysis was used to quantify the neurite outgrowth on unmodified and charge modified hydrogels. The distribution of neurite lengths from DRG explants cultured on the different hydrogel formulations and on laminin-derived peptide (LDP) coated plastic appears in Fig. 5. In these histograms, the y-axis, labelled “frequency”, represents the number of neurites of a given length, and N is the total number of DRG explants used for the neurite length measurement. These data show that the neurite distribution in the DRG explants on charged hydrogels was shifted to the right (longer neurites) compared to the unmodified hydrogel. The neurite outgrowth on charged hydrogels was distributed more uniformly, and the median neurite lengths on charged hydrogels and LDP-coated plastic were significantly higher than those on the unmodified hydrogel. DRG neurite lengths on different hydrogel formulations and LDP-coated plastic are compared in Fig. 6, which shows that neurite lengths on charged hydrogels and LDP coated plastic after 24 and 40 hours were significantly higher than those on unmodified hydrogel (HG-0) ($p < 0.01$). Fig. 6 also shows that there was a significant increase in neurite length on both HG-400 and HG-600 compared to LDP coated plastic at 40 hours ($p < 0.01$). However, with increase in charge concentration to 800mM (HG-800) the neurite lengths significantly decreased as compared to HG-400 and HG-600.

3.3. Dissociated DRG Cell Attachment and Neurite Extension

Dissociated DRG cells were seeded onto a series of hydrogels that had different MAETAC concentrations. As shown in Figure 7, cells attached to a greater extent and extended more neurites on the surface of the positively charged hydrogel compared to the unmodified hydrogel.

3.4. In Vitro Myelination

In addition to neurite extension, myelin formation by Schwann cells was investigated using rat DRG neuron and Schwann cell co-cultures as described by Wood et al [19]. After three weeks of culture, both Schwann cells and DRG neurons remained viable, and the DRGs extended neurites. Sudan black staining revealed that neurites emerging from the DRGs were accompanied by migrating Schwann cells, which were aligned along the neurites and differentiated into myelinating Schwann cells. Multiple myelinated internodes (white arrows) and nodes of Ranvier (circles) are seen in Fig. 8.

4. Discussion

The aim of this study was to develop charge-modified hydrogels with properties that could be varied in a controlled manner, and to provide an appropriate substrate for nerve cell attachment and differentiation. OPF macromer, copolymerized with varying amounts of a pH-dependent cationic monomer (MAETAC), becomes a hydrogel that contains a permanent charge. The amount of that charge depends on the MAETAC concentration in the polymerization recipe, and we determined the MAETAC's effect on both the surface and mechanical properties of the hydrogels. Neuronal cell anchorage to the hydrogels, neurite extension from the neuronal cells, and Schwann cell differentiation, migration, and myelination in vitro were analyzed as specific responses to the changes in the hydrogel charge densities. Primary sensory rat DRG neurons attached themselves to the charge-modified hydrogels and extended neurites. DRG explants that contained sensory neurons, Schwann cells, and glial cells demonstrated the attachment of the neuronal cells and the supporting cells that are critical for neural tissue regeneration. Neurite lengths increased with increasing MAETAC concentration at both 24 and 40 hours of culture.

Attenuated total reflectance FTIR data demonstrated that MAETAC was successfully incorporated into the OPF hydrogel. The amount of MAETAC copolymerized within the OPF hydrogel linearly increased with increasing MAETAC concentration in the hydrogel precursor solution (Fig. 2C). After hydration in deionized water, the carbon-oxygen (C-O) peak intensity decreased on hydrogel surfaces as the concentration of positively charged monomer increased. However, when the hydrogels were hydrated in PBS, the carbon-oxygen (C-O) peak intensity on the charge-modified hydrogels, although less than that in the unmodified hydrogels, was greater than it was when hydration occurred in deionized water (Fig. 3B). The difference in C-O peak intensities between hydrogels that were hydrated in deionized water or PBS was associated with decreased swelling ratios of charge-modified hydrogels in PBS compared to those in deionized water. The change in C-O peak intensity associated with varying swelling ratios in either water or PBS is consistent with the ionic nature of these charge-modified hydrogels. Furthermore, the incorporation of the unsaturated MAETAC monomer increased the frequency of crosslinks between OPF polymer chains, as demonstrated by a decreased sol fraction of charge-modified OPF hydrogels compared to unmodified hydrogels. This decrease in the sol fraction is indicative of a highly crosslinked network (Table 1), and accounts for the increase in the compressive modulus of the charge-modified hydrogels compared to the unmodified hydrogels. The swelling ratios of the charge-modified hydrogels in PBS remained unchanged with increasing MAETAC concentration in the hydrogel formulations. We hypothesize that there is an equilibrium state for absorption of PBS due to the presence of ionic moieties in the hydrogel backbone. This equilibrium state could be associated with the changes in osmotic pressure within the hydrogel network. These findings are in agreement with contact angle data that charge-modified hydrogels became more hydrophobic with increasing MAETAC concentration in the hydrogel formulations when the hydrogels were swollen in deionized H₂O (data not shown).

Charge-modified hydrogels supported attachment and neurite outgrowth from DRG explants containing Schwann cells in addition to enhancing those same processes from primary sensory rat (DRG) neurons (Fig. 5). The neurite lengths for DRG explants grown on HG-400 (987 ± 128 μ m) and on HG-600 (1175 ± 88 μ m) were significantly higher than those on unmodified hydrogel (0 ± 0 μ m) and LDP coated plastic (389 ± 141 μ m) after 40 hours (Fig. 6). However, with increase in charge concentration to 800mM (HG-800) the neurite lengths significantly decreased as compared to HG-400 and HG-600. These findings suggest that charged OPF hydrogels are capable of sustaining both the primary nerve cells and the support cells that must be present for neural tissue regeneration. Previous studies have shown that polycationic polymers such as polylysine and polyornithine [21–24] improved cell attachment and neurite

elongation in vitro when they were used as coating materials. These previous studies have shown that the presence of the local positive charge improves both cell adhesion to the substrate and neurite outgrowth. Other investigators have reported the repair of transected sciatic nerves in adult mice by charged guidance channels [25]. The nerves regenerated in positively charged channels contained significantly more myelinated axons than those in negatively charged or uncharged channels. The present study also suggests a general relationship between positive electrical charges and the enhancement of neurite outgrowth. Although the mechanism for the biologic effects of the positive electrical charge is not clear, it has been suggested that a permissive range of calcium ion concentration is required for optimal neurite outgrowth [26]. Since the influx of calcium ions across the cell membrane is regulated by the voltage-gated Ca^{2+} channels, interactions with an electrically charged substrate may lead to the changes in the ionic flux. Thus, MAETAC hydrogels may change calcium influx across the neuronal membrane, which could then be responsible for the enhancement of neurite outgrowth. Changes in calcium concentration may also influence the interaction with calmodulin, activate protein kinase C, or directly affect the activity of intercellular enzymes [26]. Other signal transductive candidates are membrane receptors whose interactions with G-proteins are modified by electrical stimulation. Additional events that may be influenced by electric fields include mitosis of Schwann cells, increased macrophage activity, upregulation of trophic factor production, increased axonal transport, and basal lamina production.

We demonstrated that charge modification of the OPF hydrogels improved neuronal cell attachment to the hydrogels in a dose dependent manner. Figure 7 shows that few DRG cells were attached to the unmodified OPF hydrogels, and that they attached to a greater extent on the charged hydrogel surfaces. A dense network of neurites emerging from DRG neurons was observed on the HG-400 charge-modified hydrogel after 7 days in culture, indicating that the neurons were better able to express their phenotypic function on the charge-modified hydrogels than on the unmodified hydrogels (Fig. 7).

A co-culture of charged hydrogels, DRG neurons and Schwann cells was analyzed for the appearance of myelinated structures. In the peripheral nervous system, differentiated Schwann cells produce myelin, which consists of highly specialized plasma membrane sheaths. Synthesis, deposition, and organization of specific myelin components require an orchestrated series of cellular events to generate and maintain the myelin sheath around the axon [27,28]. In vitro myelination has been previously described on both collagen I and poly(L-lactide) [20,29]. Here, we demonstrated that after 3 weeks in culture, both DRGs and Schwann cells remained viable on charged hydrogels (HG-400), and that the DRGs extended neurites. Sudan black staining revealed that emerging neurites from DRGs were accompanied by migrating Schwann cells, which were aligned along the neurites and differentiated into myelinating Schwann cells. In addition, multiple myelinated internodes and nodes of Ranvier were seen over the myelinated axons (Fig. 8).

5. Conclusion

The present study demonstrates that modification of photocrosslinkable OPF with a positively charged monomer improves DRG neuron attachment and differentiation in a dose-dependent manner. Positively charged hydrogels also supported attachment of DRG explants that contain neurons, Schwann cells and the neuronal support cells that are critical for neural tissue regeneration. Neurite extension was observed shortly after culturing the DRG explants on charged hydrogels, and the neurites grew longer with time. Neurite lengths on charged hydrogels were significantly greater than those on control groups. Moreover, charged hydrogels supported both the viability and differentiation of the neurons and Schwann cells in co-culture for a time period of three weeks. Schwann cells in culture differentiated to a myelinating phenotype, and they formed myelin sheaths on newly formed DRG axons. These

findings suggest that the presence of positive charge on a neuronal cell attachment surface is an important factor in the subsequent behavior of the cells that anchor on that surface. The charged OPF hydrogels described in this study represent attractive candidate scaffolds for neural tissue engineering applications.

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Fig. 1. Schematics of oligo-(polyethylene glycol) fumarate (OPF) charge modification and crosslinking. The OPF (A), composed of repeating PEG and fumarate chains, was crosslinked with [2-(methacryloyloxy) ethyl]-trimethylammonium chloride (MAETAC) (B) in the presence of the photoinitiator Irgacure 2959 and ultraviolet light to form positively charged hydrogels.

Fig. 2.

Micro-ATR-FTIR of hydrogels after crosslinking and lyophilization: unmodified hydrogel (A), and charged hydrogels with different MAETAC concentrations (B). With increasing MAETAC concentration in hydrogel formulations, the peak intensity at 1725 cm⁻¹ assigned to methacroyl group from MAETAC increased, and the peak intensity at 1650 cm⁻¹ assigned to carbonyl from OPF decreased. The linear relationship between the amounts of MAETAC added to the OPF solution and the levels of covalently coupled MAETAC in crosslinked hydrogels appears in panel (C). The solid line is a linear fit of the data ($R^2=0.9905$).

Fig. 3.

Hydrogel (HG-400) spectra after hydration in PBS and deionized water (A). Peaks at 1725 and 1650 cm^{-1} merge together after hydration in either PBS or water and appear at 1650 cm^{-1} . Peak intensities of the C-O bond at 1085 cm^{-1} vary with the change in MAETAC concentration as well as with the choice of either water or PBS as solvent. Comparisons of 1085/1650 peak ratio on the hydrogel surfaces as a function of MAETAC concentration after hydration in either deionized water or PBS are shown in panel (B). The decrease in C-O peak intensity (1085 cm^{-1}) correlates with an increase in MAETAC concentration when the hydrogels are swollen in water. This change occurs to a lesser degree when the hydrogels are swollen in PBS.

Fig. 4. Phase contrast images of DRG explants (containing neurons, Schwann cells and supporting cells) cultured for 40 hours on either a charge-modified hydrogel (HG-400, A) or an unmodified hydrogel (HG-0, B). Note that the purple color seen in this image is due to up-taking the phenol-red from media by positively charged hydrogel. There are many neurites extending from the DRG explant on the charge-modified hydrogel, and no neurites from the DRG on the unmodified hydrogel. All magnifications are $\times 25$.

Fig. 5.
Histograms of neurite lengths of DRG explants cultured for 40 hours.

Fig. 6. Influence of charge modification on DRG explant neurite extension after 24 and 40 hours in culture. Data represent mean $\pm$ SEM (n=8–16). (*) p<0.01 compared to HG-0 after 24 hours. (+) p<0.01 compared to HG-0 after 40 hours. (#) p<0.01 compared to LDP-coated plastic after 40 hours.

Fig. 7. Phase contrast images of DRG neurons after 7 days culture on an unmodified hydrogel (HG-0) (A), and on a charge-modified hydrogel (HG-400) (B) after treatment with 5-fluorodeoxyuridine (FUDR) to remove Schwann cells. Cells on the unmodified hydrogel do not attach and they tend to aggregate, while cells on the charge-modified hydrogel attach and extend neurites. All magnifications are $\times 200$.

Fig. 8. Sudan black stained neurons and Schwann cells after three weeks in culture. There was no FUDR treatment after plating, which permitted Schwann cell growth and differentiation on the newly formed neurites. There are multiple myelinated internodes (arrows) and nodes of Ranvier (circles).

Table 1
Swelling ratios and sol fractions of unmodified and charge-modified OPF hydrogels

Hydrogel	MAETAC Concentration (mM)	Equilibrium Swelling Ratio			Sol Fraction (%)	Compressive Modulus (kPa)
		Water	PBS			
HG-0	0	6.2±0.2	6.8±0.3		15.7±0.8	225±20
HG-200	200	7.6±0.1 [*]	6.2±0.1 [*]		10.6±1.4 [*]	331±25 [*]
HG-400	400	8.7±0.2 [*]	6.2±0.1 [*]		8.0±3.0 [*]	310±23 [*]
HG-600	600	9.2±0.2 [*]	6.1±0.2 [*]		9.1±2.6 [*]	324±28 [*]
HG-800	800	9.6±0.2 [*]	5.9±0.1 [*]		7.3±0.6 [*]	313±15 [*]

* significant at the p=0.05 level compared to HG-0